CHAPTER 3

SDHAF1, encoding a LYR complex-II specific assembly factor, is mutated in SDH-defective infantile leukoencephalopathy

Daniele Ghezzi[1], *Paola Goffrini*[2], *Graziella Uziel*[3], *Rita Horvath*[4,5], *Thomas Klopstock*[5], *Hanns Lochmüller*[5,6], *Pio D'Adamo*[7], *Paolo Gasparini*[7], *Tim M Strom*[8], *Holger Prokisch*[8], *Federica Invernizzi*[1], *Ileana Ferrero*[2] *& Massimo Zeviani*[1]

1. Unit of Molecular Neurogenetics–Pierfranco and Luisa Mariani Center for Study of Children's Mitochondrial Disorders, Foundation IRCCS Neurological Institute "C. Besta", Milan, Italy. 2. Department of Genetics, Anthropology, Evolution, University of Parma, Parma, Italy. 3. Department of Child Neurology, Foundation IRCCS Neurological Institute "C. Besta", Milan, Italy. 4. Mitochondrial Research Group, Newcastle University, Newcastle upon Tyne, UK. 5. Department of Neurology, Friedrich-Baur Institute, Ludwig-Maximilians University, Munich, Germany. 6. Institute of Human Genetics, Newcastle University, Newcastle upon Tyne, UK. 7. Department of Medical Genetics, IRCCS Burlo Garofolo, University of Trieste, Trieste, Italy. 8. Institute of Human Genetics, Helmholtz Zentrum, Munich, Germany.

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We report mutations in *SDHAF1*, encoding a new LYR-motif protein, in infantile leukoencephalopathy with defective succinate dehydrogenase (SDH, complex II). Disruption of the yeast homolog or expression of variants corresponding to human mutants caused SDH deficiency and failure of OXPHOS-dependent growth, whereas SDH activity and amount were restored in mutant fibroblasts proportionally with re-expression of the wild-type gene. SDHAF1 is the first bona fide SDH assembly factor reported in any organism.

Succinate dehydrogenase (SDH, or complex II, cII) is composed of four subunits (SDHA-D)[1], all encoded by nuclear genes. The two larger subunits, SDHA and SDHB, are catalytic. Dehydration of succinate to fumarate is accomplished by SDHA through reduction of a flavin-mononucleotide (FMN) molecule bound to its protein moiety. This reaction is measured as succinate dehydrogenase (SDH) activity. Electrons are then passed to three Fe-S centers bound to SDHB, which eventually transfers them to ubiquinone (coenzyme Q, coQ). The latter reaction is measured as succinate-CoQ reductase (SCoQR) activity. The smaller subunits, SDHC and SDHD, anchor the complex to the inner membrane of mitochondria. Heterozygous mutations in *SDHB, SDHC* and *SDHD* are responsible for dominantly inherited paragangliomas and phaechromocytomas[2, 3, 4]. In our series of subjects with infantile mitochondrial disease, 22/280 (8%) had a specific biochemical defect of cII. Nevertheless, only four 'private' mutations, all affecting *SDHA*, have ever been reported, in three families with cII-associated Leigh syndrome[5, 6, 7].

Here we studied two family sets (Supplementary Fig. 1a online), one consisting of a large multiconsanguineous kindred of Turkish origin with several affected children, the other composed of three affected children and their parents, originating from a small village in an alpine valley of Lombardy, Italy. Two of the Italian affected children were second-degree cousins, one being born from first-degree cousin parents. Although we failed to formally ascertain the consanguinity of the other parents and to connect the family of the third child with the other two, we assumed that all affected individuals had inherited by descent the same, presumably homozygous, mutation on the basis of virtually identical clinical presentations and common geographic origin.

The clinical features of the Turkish and Italian subjects were very similar (Supplementary Table 1 online) and have partly been described elsewhere[8, 9]. Symptoms consisted essentially of rapidly progressive psychomotor regression after a 6- to 11-month disease-free interval with lack of speech development, followed by spastic quadriparesis and partial loss of postural control with dystonia. Brain magnetic resonance imaging showed severe leukodystrophic changes with sparing of the peripheral U-fibers and basal ganglia. Proton magnetic resonance spectroscopy revealed a decreased *N*-acetyl-aspartate signal and abnormal peaks corresponding to accumulation of lactate and succinate in the white matter[8, 9]. Lactate and pyruvate were variably elevated in blood. The subjects underwent relative stabilization of their clinical conditions, with survival beyond the first decade of life in several cases, although their growth was consistently and severely impaired. Biochemical analysis of mitochondrial

respiratory chain (MRC) complexes in muscle and fibroblasts showed a ~20–30% residual activity of SDH and SCoQR, whereas the other MRC activities were normal (Supplementary Table 2 online). Protein blot analysis on one- and two-dimensional blue-native gel electrophoresis showed marked reduction of cII holoenzyme in muscle (Fig. 1a) and fibroblasts (Fig. 1b).

The methodological procedures used in the experimental workout are reported in Supplementary Methods online. Genome-wide linkage analysis using SNP array genotyping in the Turkish family identified a 13.5-Mb homozygous region on chromosome 19q12–q13.2 between rs9304866 and rs2317314 with a maximal lod score of 5.7. Concordant results were independently obtained by SNP-based mapping of the Italian families, but here the region of continuous homozygosity was only 1.2 Mb, between recombinant markers rs3761097 and rs2562604, which contains 42 annotations (Supplementary Table 3 online). A single anonymous entry in the region, termed *LOC644096*, consisting of a single exon, predicts the translation of a 115-amino-acid protein sequence (NP_001036096), which scores high when analyzed by mitochondrial targeting prediction programs (Supplementary Table 3). We found two homozygous missense mutations in *LOC644096*—which will from now on be termed *SDHAF1*, for SDH assembly factor 1—segregating with the disease: 169G>C, corresponding to G57R in the Italian individuals, and 164G>C, corresponding to R55P, in the Turkish individuals (Supplementary Fig. 1b). The mutant amino acid positions are highly conserved across species (Supplementary Fig. 1c). We found no *SDHAF1* mutations in 20 individuals with cII deficiency

with other clinical presentations or in 660 European and 150 Turkish consecutive healthy control subjects.

To establish whether the SDHAF1 protein is targeted to, and resides within, mitochondria, we expressed a hemoagglutinin-epitope (HA)-tagged recombinant protein in COS7 cells and found that the HA-specific immunofluorescence pattern coincides with that of mtSSB, a mitochondrial-specific marker protein (Fig. 1c). We then found by *in vitro* import assay that the SDHAF1 protein is translocated by the proton motive–dependent transport system into the inner mitochondrial compartment, where it is protected from digestion with proteinase K[10]. The size of the *in vitro* translated product corresponding to the full-length *SDHAF1* gene ORF is identical to that of the imported polypeptide (Fig. 1d), indicating that the protein does not undergo post-import cleavage of the N-terminal mitochondrial targeting sequence. We observed no difference in *in vitro* mitochondrial translocation between wild-type and mutant SDHAF1 species (Supplementary Fig. 2 online). The *SDHAF1* gene transcript is ubiquitously expressed (Supplementary Fig. 3a online) and is translated into a relatively hydrophilic protein with no predicted transmembrane domain (Supplementary Fig. 3b), which suggests that it resides in the mitochondrial matrix. This hypothesis was confirmed experimentally by protein blot analysis on subcellular fractions of $SDHAF1^{HA}$-expressing HeLa cells. Thus, albeit essential for SDH biogenesis, SDHAF1 is not physically associated with cII *in vivo* (Fig. 1e).

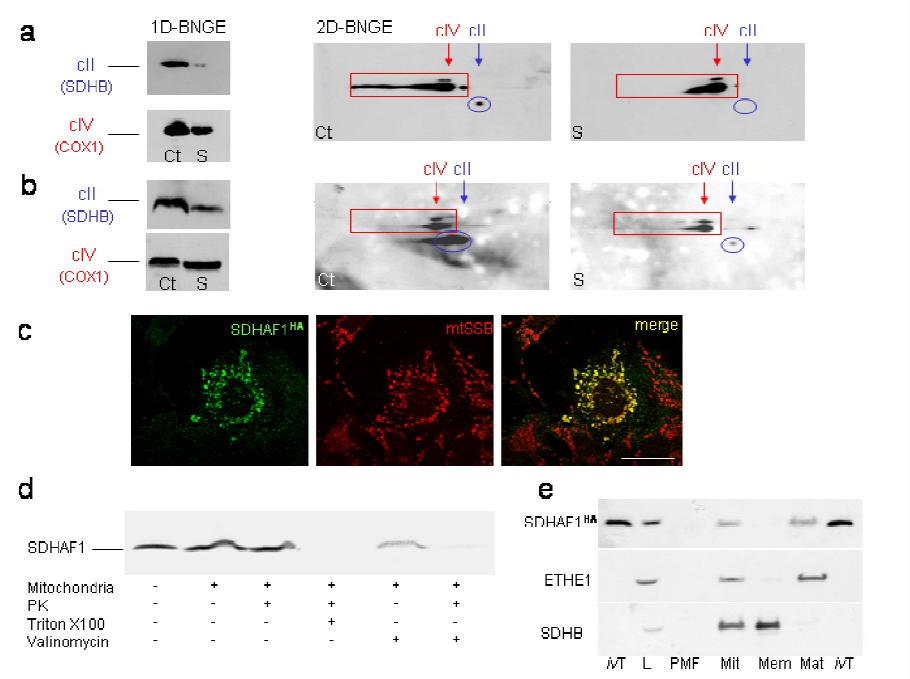

Figure 1: Protein blot and immunofluorescence studies.

(**a**,**b**) One- and two-dimensional blue-native gel electrophoresis (1D- and 2D-BNGE) protein blot analysis from subject 5 (S) and a control (Ct) in muscle (**a**) and fibroblast homogenates (**b**). SDHB and COX1 are subunits of SDH (blue circle) and COX (red square) holoenzymes. (**c**) Confocal immunofluorescence of COS7 cells transfected with $SDHAF1^{HA}$/pCDNA3.2. Scale bar, 30 μm. (**d**) *In vitro* import assay. (**e**) Protein blot analysis of HeLa cell fractions expressing $SDHAF1^{HA}$. *iv*T, *in vitro* translated $SDHAF1^{HA}$; L, cell lysate; PMF, postmitochondrial fraction; Mit, mitochondrial fraction; Mem, mitochondrial membrane fraction; Mat, mitochondrial matrix. Antibodies to a mitochondrial matrix protein (ETHE1) and an inner-membrane protein complex (SDHB) were used as markers.

To test whether the disease-segregating missense mutations of *SDHAF1* are indeed causing cII deficiency, we first used a *Saccharomyces cerevisiae* system. We disrupted the *YDR379C-A* gene, the yeast ortholog of *SDHAF1*, by homologous recombination (Supplementary Fig. 4a online). The Δ*ydr379c-a* yeast strain was OXPHOS incompetent because of a profound and specific reduction of cII activity, whereas complex IV (cIV, cytochrome *c* oxidase,

COX) activity was normal (Fig. 2a). Transformation with the wild-type *YDR379C-A*, but not with *YDR379C-A* variants corresponding to the human mutant species, restored OXPHOS growth of the Δ*ydr379c-a* strain (Fig. 2a). Expression of wild-type human *SDHAF1* also failed to complement the yeast strain (Supplementary Fig. 4b), possibly because of the low similarity between yeast and human protein species. Respiration in standard YBN medium containing 0.6% glucose was only slightly reduced, and cytochrome spectra were normal (Fig. 2a), indicating the integrity of the other components of MRC. The apparent K_m value for succinate was 0.87 mM in wild-type and 0.85 mM in the null mutant, suggesting that defective SDH activity is caused by reduced number of enzyme units rather than by qualitative alterations of cII. We then expressed wild-type human *SDHAF1* in three G57R mutant fibroblast cell lines. SDH and SCoQR cII activities were completely recovered in cell line 1, whereas cell line 2 showed partial recovery (80%) as did cell line 3 (40%) (Fig. 2b). The content of the recombinant *SDHAF1* wild-type RNA was proportional to the recovery of enzymatic activity (Fig. 2b), which was paralleled by increased content of fully assembled cII (Fig. 2c). Taken together, our results demonstrate that (i) mutations in *SDHAF1* cause an isolated cII defect associated with a specific leukoencephalopathic syndrome and (ii) the SDHAF1 product is the first bona fide assembly factor specific to cII, as its loss determines severe reduction in the amount of the enzyme in both yeast and humans.

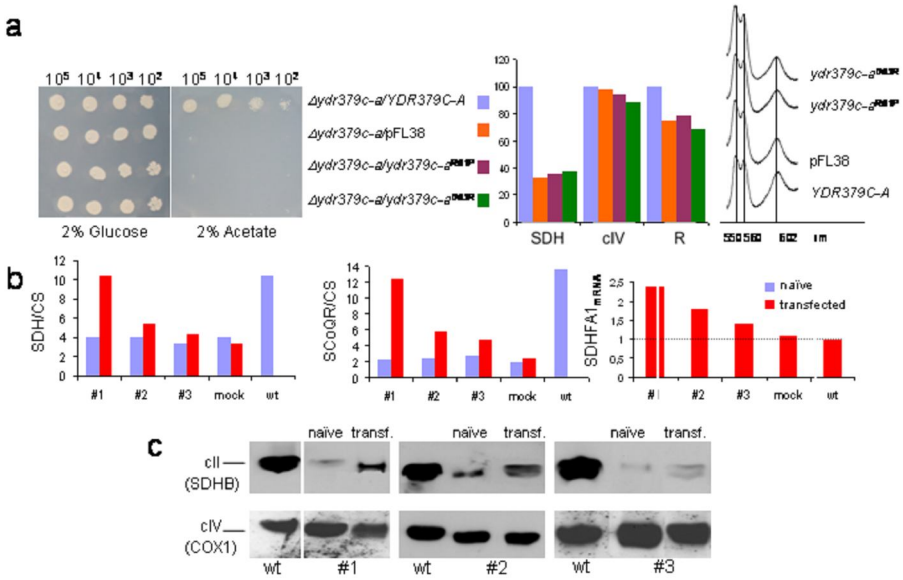

Figure 2: Complementation assays in yeast and human cells.

(**a**) Results on *YDR379C-A* deleted yeast strain (Δ*ydr379c-a*). Left, OXPHOS growth. The Δ*ydr379c-a* strain was transformed with wild-type *YDR379C-A* allele; pFL38 empty vector; and *ydr379c-a*^{R61P} or *ydr379c-a*^{G63R} mutant alleles. Middle, biochemical assays. Biochemical activities (nmols per min per mg protein) of SDH and COX normalized to that of citrate synthase (CS). Respiration (R) was measured as nmol O_2 per min per mg dry weight. All values are expressed as percentage of the activities obtained in the control strain Δ*ydr379c-a/YDR379C-A*. Right, reduced versus oxidized cytochrome spectra. Peaks at 550, 560 and 602 nm correspond to cytochromes *c*, *b* and *aa3*, respectively. The height of each peak relative to the baseline is an index of cytochrome content. (**b**) Biochemical and molecular characterization of fibroblasts cell lines. Left, SDH/CS (normal range 6.5–14.3). Middle, SCoQR/CS (normal range 8.6–18.4). Right, ratio of total/endogenous *SDHAF1* mRNA. WT, wild-type control cell lines; cell lines 1, 2 and 3, G57R mutant cell lines transfected with *SDHAF1/pCDNA3.2* vector; mock, G57R mutant cell line transfected with empty vector. (**c**) Protein blot analysis on one-dimensional blue-native gel electrophoresis. SDHB and COX1 are subunits of SDH and COX holoenzymes. N, naïve; T, transfected.

SDHAF1 contains a LYR tripeptide motif, which is present in the N-terminal region of several protein sequences in different species. There are at least eight LYR-motif (LYRM) proteins in humans, including SDHAF1. LYRM-4 is the human ortholog of yeast ISD11, a protein that has an essential role in the mitochondrial biosynthesis of

Fe-S centers[11]. LYRM-6 is the 14 kDa NDUFA6 subunit of complex I (cI)[12]; a second cI subunit, the 22 kDa NDUFB9 is also a LYR, iron-responsive protein[13]. These data suggest that the LYR motif is a signature for proteins involved in Fe-S metabolism. In particular, NDUFA6, NDUFB9 and possibly SDHAF1 as well could be important for the insertion or retention of the Fe-S centers within the protein backbones of cI and cII, respectively. Failure of the Fe-S centers to be incorporated within cII may eventually inhibit the formation or destabilize the structure of the holocomplex. Although there are other examples of low cII content and activity associated with mutations in mitochondrial chaperonins such as yeast Tcm62.p[14], or proteins involved in Fe-S biosynthesis such as human and yeast frataxin or IscU[15], SDHAF1 is the only protein so far identified with a specific role for cII, as other Fe-S–dependent activities were normal in SDHAF1-defective organisms, including cI in humans and complex III in both humans and yeast.

Acknowledgments

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Author Contributions

D.G. found *SDHAF1* and characterized the mutations in human cells; P.G. and I.F. carried out the experiments in yeast; G.U., R.H., T.K. and H.L. identified the subjects and carried out the clinical workout; P.D., P.G., T.M.S. and H.P. performed linkage analysis on the Italian and Turkish family sets; F.I. carried out the biochemical assays on subjects and the mutational screening on family members, disease and healthy controls; and M.Z. conceived the experimental planning and wrote the manuscript.

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Supplementary Information

SUPPLEMENTARY METHODS

Genetic studies

Genome-wide genotyping in the Turkish family was performed using Human Mapping 10K Arrays from Affymetrix (Xba142 version 2) which contain approximately 10,000 SNP markers. Frequencies of marker alleles were derived from dbSNP as provided by Affymetrix. Data for linkage analysis were prepared with a modified version of Alohomora (Ruschendorf, F. et al. Bioinformatics 21, 2123-2125 (2005)). Multipoint linkage analysis was performed using Allegro version 1.1d (Gudbjartsson, D.F. et al. Nat. Genet. 25, 12-13 (2000)). We assumed an autosomal recessive model. The frequency of the deleterious allele was set to 0.001, and the penetrance to 99% (q= 0.001; f1=0.0; f2=0.0; f3=0.99).

Genome-wide genotyping in Italian patients was performed using ILLUMINA 370CNV arrays. Regions of homozygosity were searched for by using a function implemented in the software PLINK (http://pngu.mgh.harvard.edu/~purcell/plink/index.shtml).

Mutation screening

This study was approved by the local Ethics Committee. Each participant signed an informed consent prior to participation in the study.

Blood samples were collected from parents, patients and control subjects; genomic DNA was extracted by standard methods. PCR

amplification of the exon 1 of SDHAF1 was obtained using the following primers: Fw: ACAGCCAGTCAGACGCGG; Rc: CACCTTAGGCGGGTTCCC.

The amplicons were sequenced with both forward and reverse primers by PCR cycle sequencing (Big Dye Terminator System) and analysed on an ABI Prism 3130XL Genetic Analyzer (Applied Biosystems). To analyze the control cohort, RFLP analysis was performed using *Bsl*I, for mutation c.164G-C, and *Nae*I, for c.169G-C (NewEngland Biolabs).

Total RNA was isolated from cultured fibroblasts (Rneasy kit, Qiagen) and then transcribed to cDNA (Cloned AMV first-strand cDNA synthesis kit, Invitrogen). Quantitative Real-time PCR was assayed on an ABI Prism 7000 apparatus (Applied Biosystems). We used primers specific for the coding sequence of SDHAF1 and primers for a specific 3'-UTR sequence, present in the endogenous transcript but not in the SDHAF1 expression vector (available upon request). The amount of total SDHAF1 cDNA was normalized to that of the endogenous cDNA.

Biochemical assays

Biochemical analyses were performed on the 800xg supernatants obtained from crude muscle homogenates and on digitonin-treated cultured skin fibroblasts. Enzyme activities of respiratory chain complexes were measured spectrophotometrically and normalized to that of citrate synthase, as described by Bugiani, M. et al. (Biochim. Biophys. Acta 1659, 136-147 (2004)).

Assembly state analysis

The detection of the assembled respiratory complexes in control and patient skin fibroblasts, as well as in muscle biopsies, was performed by using BNGE. Samples were obtained from 1-2 x 10^6 cultured fibroblasts or 25 mg of muscle biopsy, as described by Nijtmans, L.G. et al. (Methods 26, 327–334 (2002)), with slight modifications. Fifteen to twenty microliters of the sample was loaded and run into a 5-13% gradient non-denaturating 1D-BNGE. For denaturing 2D-BNGE electrophoresis, the 1D-BNGE lane was excised, treated for 1 h at room temperature with 1% SDS and 1% b-mercaptoethanol and then run through a 16.5% tricine-SDS–polyacrylamide gel, using a 10% spacer gel (Schagger, H. et al. Anal. Biochem. 166, 368–379 (1987)).

Cloning of MR-1 cDNA

Full ORFs of the wt and mutant SDHAF1 were obtained from genomic DNA of control subject and patients. The full-length transcripts were also 3'-tagged with the sequence encoding an epitope of the haemoagglutinin (HA) of the influenza virus.

Primers (forward, Fw; reverse complementary, Rc):

SDHAF1 Fw:

CACCATGAGCCGGCACAGCCGGCTGCAGAGGCA;

SDHAF1 wt Rc:

CGGTTTATTCGGTCACCGTCCGTCGGGGCGGGTCTCCGGTG

SDHAF1 HA Rc:

CGGTTTATTCGGTCAAGCGTAATCTGGAACATCGTATGGGT

ACCGTCCGTCGGGGCGGGTCTCCGGTGC.

The constructs were inserted into the eukaryotic-expression plasmid vector pcDNA3.2 (Invitrogen). The resulting vectors were named SDHAF1/pCDNA3.2 and SDHAF1^{HA}/pCDNA3.2.

Cell Culture and Transfection of COS7 Cells and mutant fibroblasts

Monkey kidney (COS7) cells, HeLa cells and skin fibroblast from patients were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 mg/ml streptomycin, 100 units/ml penicillin, and 1 mM sodium pyruvate at 37°C in a humidified incubator containing 5% CO2. The recombinant plasmids were transfected by electroporation in both COS7 cells (for transient expression) and HeLa cells or fibroblasts from patients (for stable expression). Stably transfected clones of fibroblasts were selected under 100ng/ml Geneticin (G418; Gibco-Invitrogen.).

Immunofluorescence Studies

24-48h after electroporation, cells were plated on coverslips, fixed with 4% paraformaldehyde in PBS and lysed with PBS, 0.5% Triton X-100. After blocking with NGS 15%, cells were incubated with primary antibodies (mouse monoclonal anti-HA, Roche, and polyclonal anti-mitochondrial single-strand binding protein, mtSSB (Tiranti, V. et al. Am. J. Med. Genet. 63, 1609-1621 (1998)) in PBS, NGS 15%. Cells were washed with PBS three times each and incubated with fluorescent-dye–conjugated secondary antibodies (AlexaFluor, Molecular Probes), washed and analyzed with a Biorad confocal microscope.

Mitochondrial Import Assay

Mitochondria from HeLa cells, freshly isolated as described elsewhere (Fernandez-Vizarra, E. et al. Methods 26, 292-297 (2002)), were used to assay protein import. ^{35}S radiolabeled in-vitro translated products corresponding to full-length open reading frames (ORF) were obtained with TNT Quick Coupled Transcription/Translation Systems (Promega). After incubation with freshly isolated mitochondria at 37°C in the presence or absence of 20 µM valinomycin, samples were washed and electrophoresed through a 14% SDS-polyacrylamide gel. After fixation in 10% acetic acid and 25% isopropanol, the gel was washed for 20 min in Amplify reagent (Amersham) and layered onto a phosphorimaging screen (Biorad). After overnight exposure autoradiography was carried out in a Molecular Imager apparatus (Biorad).

Cell Fractionation

Standard methods were used for the preparation of mitochondrial enriched/postmitochondrial fractions in transfected cultured cells (Fernandez-Vizarra, E. et al. Methods 26, 292-297 (2002)). For suborganellar localization of HA-tagged SDHAF1, mitochondria from transfected HeLa cells were resuspended in PK buffer and treated with three sonication strokes, and the membrane and soluble fractions were separated by ultracentrifugation at 100,000xg for 1 hr at 4°C.

Northern-blot analysis

Commercial human multiple tissue northern blot (FirstChoice Northern Human Blot 1, Ambion) was hybridized with a specific

probe, generated by PCR and labeled with ^{32}P-dCTP using the DECAprime II random primed DNA labeling kit (Ambion). A β-actin probe was used as a loading control. Hybridization was performed according to the protocol provided by the manufacturer. After 48h exposure, autoradiography was carried out in a Molecular Imager apparatus (Biorad).

Yeast strains and culture media

Yeast strains used were BY4742 (MATa; *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0) and its isogenic *YDR379C-A:kanMX4* mutant constructed as described below.

Cells were cultured in YNB medium [0.67% yeast nitrogen base without amino acids (ForMedium™, United Kingdom)] supplemented with 1 g/l of drop-out powder containing all amino acids except those required for plasmid maintenance. Various carbon sources were added at 2% (w/v) (Carlo Erba Reagents, Italy)**.** Media were solidified with 20g/l agar (ForMedium™, United Kingdom). YP medium contained 1% Bacto-yeast extract and 2% Bacto-peptone (ForMedium™, United Kingdom). For mitochondria extraction cells were grown to late-log phase in the YNB medium supplemented with 0.6% glucose.

Construction of yeast strains carrying ydr379c-a mutations

Saccharomyces cerevisiae strain BY4742 was used for the construction of a null □*ydr379*c-a mutant by one step gene disruption using KanMX expression cassette (Wach, A. et al. Yeast. 10, 1793-808 (1994)). *YDR379C-A* ORF was completely deleted and substituted by the Kan^{r} marker, whose correct target at *YDR379C-A*

locus was verified by PCR. Oligonucleotides used for *YDR379C-A* disruption are listed in table A.

Site-directed mutagenesis and sequence analysis

The QuikChange site-directed mutagenesis Kit (Stratagene, La Jolla, CA) was used to introduce the two different point mutations in the *YDR379C-A* gene. The *YDR379C-A* wild-type gene cloned in the pFL38 centromeric plasmid (Bonneaud, N. et al. Yeast 7, 609–615 (1991)) was used as template DNA. To obtain this plasmid, we PCR-amplified a DNA fragment of 710 bp containing the ORF and the 5' and 3' flanking regions (380bp and 100bp), using genomic DNA of strain BY4742 as template and the appropriate forward and reverse primers containing restriction sites at their 5' ends. The sequences of the oligonucleotides used for cloning and the sequences of the modified primers used to generate mutated alleles are reported in the table below. Both wild-type and mutagenized inserts were sequence-verified on both strands.

Oligonucleotides Used for Site-Directed Mutagenesis, for Cloning and Disruption

Oligonucleotide Use and Name	Sequence (5'-3')	Amino Acid Change
Site-directed mutagenesis[a]:		
YDM55F	CCACCATTGAACACTTGTTACCAGT CGGAAACAAAAAAATTGCCT	**R61P**
YDM55R	GGCAATTTTTTTGTTTCCGACTGGT	

Oligonucleotide Use and Name	Sequence (5'-3')	Amino Acid Change
	AACAAGTGTTCAATGGTGG	
YDM57F	CATTGAACACTTGTTACGGGTCCGC AACAAAAAAATTGCCACCTTTTC	**G63R**
YDM57R	GAAAAGGTGGCAATTTTTTTGTTGC GGACCCGTAACAAGTGTTCAATG	
Cloning[b] and disruption[c]		
FY379	*GAATTC*CGGTAAATGACGGCTATCC GCG	
RY379	*CTGCAG*GCAAGCATAATACAGATTT GC	
S3KF	GGAGACGCAATACCAAGAGCATGC CTAAGAGATTAAGTGGGTTACCGTA CGCTGCAGGTCGAC	
S3KR	CATATGAAAAAAGAATAAAGTAAGA AAATAGTTCAATGTATATTCGATCG ATGAATTCGAGCTCG	

[a]Base changes are underlined.

[b]Sequences in italic at the 5′ end of the primer have been added to provide the ends necessary for cloning.

[C] Sequences underlined correspond to KanMX4 cassette.

Cytochrome spectra and respiration

Differential spectra between reduced and oxidized cells of a suspension of cells at 60 mg/ml (wet weight) were recorded at room temperature, using a Cary 219 spectrophotometer. Oxygen uptake was

measured at 30 °C using a Clark-type oxygen electrode in a 1 ml stirred chamber containing 1 ml of air-saturated respiration buffer (0.1 M-phthalate-KOH, pH 5.0), 10 mM glucose (Oxygraph System Hansatech Instruments, England) starting the reaction with the addition of 20 mg of wet weight of cells as previously described (Ferrero, I. et al. Antonie Van Leeuwenhoek 47, 11–24 (1981)).

Isolation of mitochondria and enzyme assay in Saccharomyces cerevisiae

Preparation of mitochondria was carried as previously described (Ferrero, I. et al. Antonie Van Leeuwenhoek 47, 11–24(1981)). The SDH activity was expressed as nmol/min/mg protein. The Succinate Dehydrogenase DCIP assay was conducted as described (Kim, I.C. and Beattie, D.S. Eur. J. Biochem. 36, 509-518 (1973)), with minor modifications. The initial reaction rate at 600 nm was recorded after addition of mitochondria to the cuvette containing 0.15 mM dichlorophenolindophenol, 2.5 mM phenazine methosulfate, 100 mM sodium azide, 0.1 M phosphate buffer, pH 7.6, and 15 mM succinate (sodium salt) (Sigma-Aldrich, Seelze, Germany). Cytochrome c oxidase activity was measured in isolated yeast mitochondria at 30°C following the decrease of absorbance at 550 nm, because of oxidation of cytochrome c. COX activity was normalized to that of citrate synthase (Wharton, D.C. and Tzagoloff, A. Methods Enzymol. 10, 245-250 (1967)). Protein concentration was determined by the method of Bradford (Bradford, M.M. Anal. Biochem. 72, 248-254 (1976)).

Miscellaneous

Transformation of yeast strain was obtained by the lithium acetate method (Gietz, R.D. et al. Methods Enzymol. 350, 87–96 (2002)). Restriction-enzyme digestions, *Escherichia coli* transformation and plasmid extractions were performed using standard methods (Sambrook, J. and Russel, D.W. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, NY (2001)).

Biocomputational analysis

Prediction softwares for mitocondrial targeting or subcellular localization:

TargetP (www.cbs.dtu.dk/services/TargetP)

PSORT-II (psort.ims.u-tokyo.ac.jp/form2.html)

Mitoprot (ihg2.helmholtz-muenchen.de/ihg/mitoprot.html)

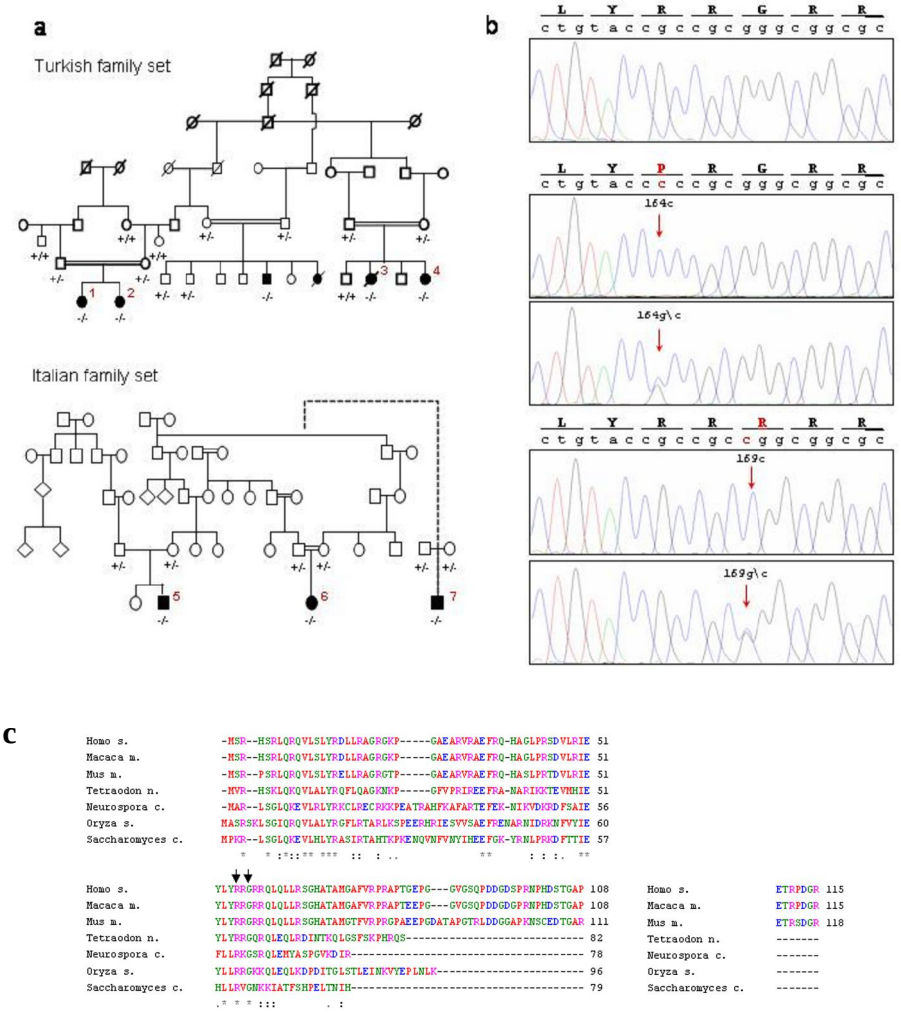
Predotar (urgi.versailles.inra.fr/predotar/predotar.html)

Prediction softwares for trans-membrane domains:

TMPRED (www.ch.embnet.org/software/TMPRED_form.html)

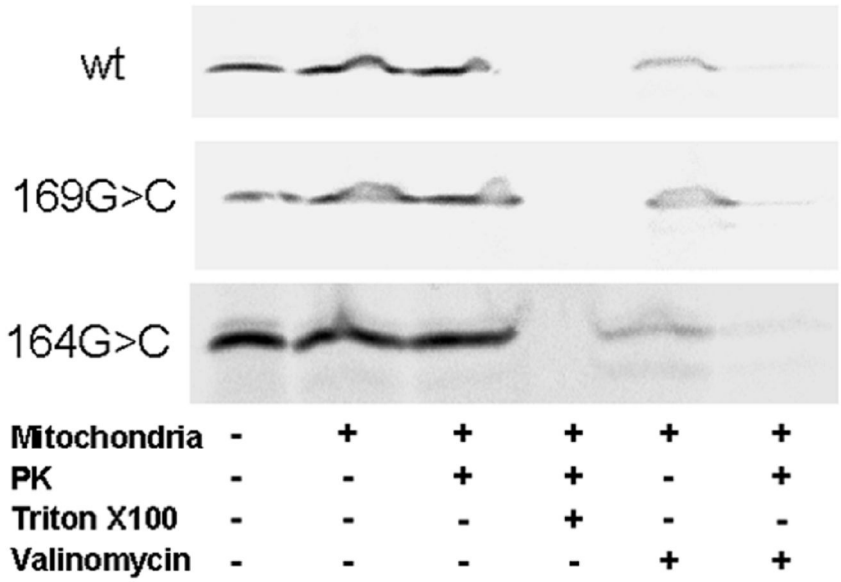
TMHMM (www.cbs.dtu.dk/services/TMHMM/)

Figure S1



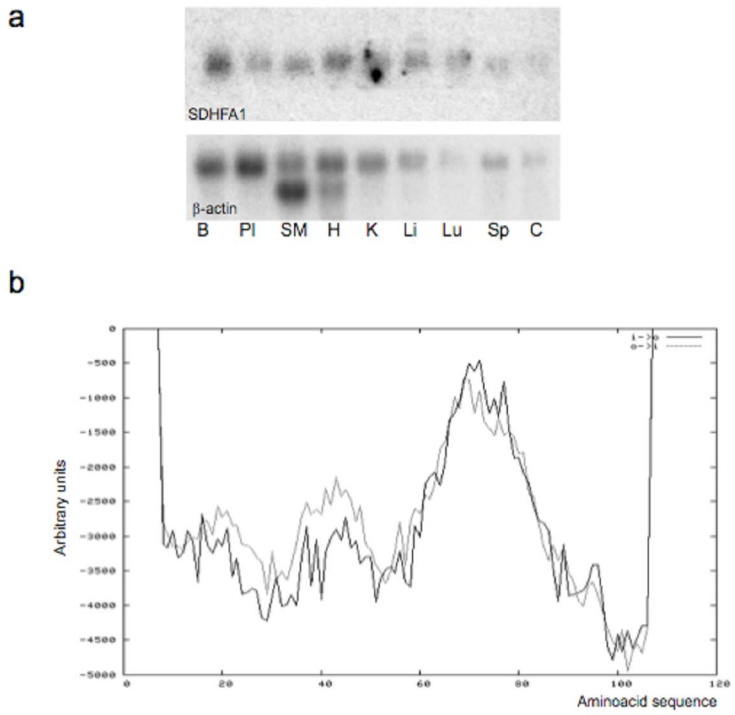
(**a**) Pedigrees of the Turkish and Italian family sets. Red numbers indicate the affected individuals who were studied. Allele genotyping symbols are +/+, for homozygous wild-type individuals, +/- for heterozygous individuals, -/- for homozygous mutant individuals. Black symbols indicate affected subjects. The dotted line indicates that the pedigree relation is suspected but not established. (**b**) Electropherograms of the genomic region encompassing the c.164G>C and the c.169G>C mutations (red arrows). (**c**) Multiple allignement of human SDHAF1 aminoacid sequence with orthologs form monkey (Macaca mulata), mouse (Mus musculus), fish (Tetraodon nigroviridis), fungi (Neurospora crassa), plant (Oryza sativa) and yeast (Saccharomyces cerevisiae), obtained with ClustalW

Figure S2



In-vitro import assay on isolated HeLa cells mitochondria of the radiolabeled full-length wild-type (wt) and mutant (containing c.169G>C or c.164G>C mutations) ORF LOC644096 products. PK: proteinase K.

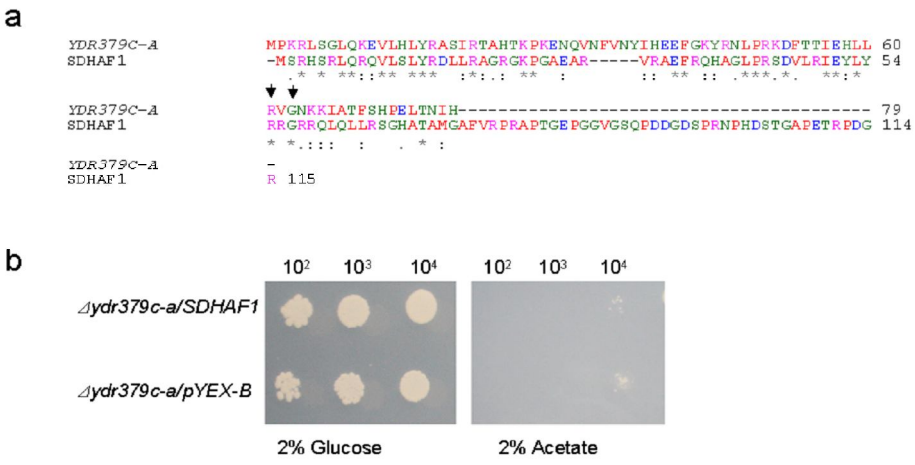
Figure S3



(**a**) Northern-blot analysis on multiple human tissues of the *SDHAF1* gene transcript encoding SDHAF1. B: brain; Pl: placenta; SM: skeletal muscle; H: heart; K: kidney; Li: liver; Lu: lung; Sp: spleen; C: colon. β-actin was used as a loading control.

(**b**) Hydropathy plot of the SDHAF1 protein sequence, generated by the TM-PRED software package. Only scores above 500 are considered as compatible with the presence of a transmembrane domain.

Figure S4.



(**a**) Alignment of human SDHAF1 aminoacid sequence with that of the yeast ortholog *YDR379C-A*.
Arrows indicate the sites of mutations found in our patients and the corresponding yeast aminoacid (human R55P-yeast R61P; human G57R-yeast G63R).
Accession numbers: NP_001036096 for human protein, NP_076888 for yeast protein.
(**b**) OXPHOS growth of yeast strains in YBN medium containing 2% glucose or 2% acetate.
Δydr379c-a/SDHAF1 is the *Δydr379c-a* transformed with human wild-type *SDHAF1*; *Δydr379c-a/pYEX-BX* is the *Δydr379c-a* transformed with plasmid without insert.

Supplementary table 1. Clinical features

Patient / Sex	Actual age	Onset	First symptom	Course	MRI / H^+MRI
1[a] / F	12 yrs	10 mo	Acute psychomotor regression triggered by febrile illness	Severe spastic quadriplegia Growth < 3° centile Moderate cognitive impairment	Leukoencephalopathy sparing U fibers. No basal ganglia involvement Succinate peak
2[a] / F	10 yrs	9 mo	Acute psychomotor regression	Severe spastic quadriplegia Growth < 3° centile Moderate cognitive impairment	Not performed
3 / F	Died 18 mo	10 mo	Acute psychomotor regression	Severe spastic quadriplegia Severe irritability Growth delay	Leukoencephalopathy sparing U fibers Succinate peak
4 / F	8 yrs	10 mo	Acute psychomotor regression	Severe spastic quadriplegia Severe irritability Growth < 3° centile	Leukoencephalopathy sparing U fibers Succinate peak
5 / M	6 yrs	6 mo	Deafness vomiting and slow psychomotor regression	Severe spastic quadriplegia Growth < 3° centile Stabilization and improvement after B2 therapy	Leukoencephalopathy with vacuolating process Lactate and succinate peaks

6[b] / F	9 yrs	10 mo	Acute psychomotor regression triggered by fever	Severe spastic quadriplegia Growth < 3° centile Stabilization and improvement after B2 therapy	Leukoencephalopathy sparing U fibers. Involvement of nuclei dentati Succinate peak
7 / M	2 yrs	11 mo	Acute psychomotor regression triggered by fever	Severe spastic quadriplegia Severe irritability Normal growth Stabilization and improvement after B2 therapy	Leukoencephalopathy sparing U fibers Succinate peak

[a] Patients previously described in Brockmann, K. et al. Ann. Neurol. 52, 38-46 (2002). [b] Patient previously described in Bugiani, M. et al. Brain Dev. 28, 576–581 (2006)

Supplementary table 2. Biochemical analysis of OXPHOS activities

Muscle	**cI/CS**	**SCoQR/CS**	**cII+III/CS**	**SDH/CS**	**cIII/CS**	**cIV/CS**	**cV/CS**	**CS**
Pt. 1	112	nd	25	nd	nd	121	nd	151
Pt. 3	153	nd	57	52	nd	143	nd	73
Pt. 5	166	13	77	31	192	170	192	96
Pt. 6	79	47	nd	53	139	114	189	171
Fibroblasts	**cI/CS**	**SCoQR/CS**	**cII+III/CS**	**SDH/CS**	**cIII/CS**	**cIV/CS**	**cV/CS**	**CS**
Pt. 3	185	32	34	38	119	144	236	93
Pt. 5	102	23	nd	62	117	128	155	139
Pt. 6	177	32	nd	51	167	200	193	155
Pt. 7	210	29	nd	62	113	181	229	81

All enzymatic activities are normalized for citrate synthase (CS) activity and expressed as percentage of the lowest control value. cII+III: succinate-cyt c oxidoreductase; nd: not determined

Supplementary table 3. Genes in the region obtained by homozygosity mapping

Gene	Protein	TargetP	Mitoprot	Psort	Predotar
PRODH2 *	PROLINE DEHYDROGENASE	79	78	70	16
NPHS1					
KIRREL2 *	kin of IRRE like 2	65	72	nd	13
APLP1					
TA-NFKBH					
HCST					
TYROBP					
LOC728326					
LRFN3					
LOC644096 *	hypothetical protein loc644096	91	31	65	45
C19ORF46					
ALKBH6					
CLIP3					
THAP8 *	THAP domain containing 8	49	23	26	12
WDR62					
LOC728361 *	hypothetical protein loc728361	60	57	22	0
POLR2I					
TBCB					
CAPNS1					
COX7A1 *	cytochrome c oxidase 7A1	91	99	78	50
ZNF565					
ZNF146					
LOC100131606					
LOC100127980					
ZP14					
ZNF545					
LOC644189					
ZNF566					
LOC728752 *	hypothetical protein loc728752	45	33	17	1

ZNF260					
ZNF529					
ZNF382					
NZNF461					
LOC100129365					
ZNF567					
LOC342892					
LOC728485					
ZNF790					
ZNF345					
DKFZp779O175					
ZNF568					

* Genes that were sequenced; for these genes, the predicted mitochondrial localization (in %) of the corresponding proteins is also shown